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The Topoisomerase I Inhibitor, Camptothecin, Inhibits Equine Infectious Anemia Virus Replication in Chronically Infected CF2Th Cells

ESTHER PRIEL,[1]†* STEPHEN D. SHOWALTER,[2] MICHAEL ROBERTS,[3] STEPHEN OROSZLAN,[3] AND DONALD G. BLAIR[1]

Laboratory of Molecular Oncology, National Cancer Institute Frederick Cancer Research and Development Center,[1] and Program Resources Inc.,[2] Frederick, Maryland 21701-1013, and ABL-Basic Research Program, National Cancer Institute Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201[3]

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Camptothecin (CPT), a topoisomerase I-specific inhibitor, was found in this study to inhibit the replication of equine infectious anemia virus (EIAV) in chronically infected CF2Th cells (designated CF2Th/EIAV). By measuring viral reverse transcriptase activity in the culture medium, we demonstrated that treatment for 1 h with noncytotoxic doses of this drug inhibited production by 32 to 52%, whereas continuous exposure to this drug resulted in an 85 to 92% inhibition. No effect on the viability or growth rate of the cells was detected in any of these treatments. Indirect immunofluorescence analysis of the CPT-treated CF2Th/EIAV cells with anti-p26 capsid protein antibodies showed 60 to 85% reduction in the immunofluorescence-positive cells following drug treatment, and radioimmunoprecipitation analysis of these cells showed a comparable decrease of the pr55^{gag} precursor protein. These data suggest that CPT acts as an anti-EIAV agent to block virus replication in the chronically infected cells.

Equine infectious anemia virus (EIAV) is a member of the *Lentivirinae* subfamily of retroviruses (2, 8). This group also includes the causative agent of AIDS, the human immunodeficiency virus (HIV) (3, 8). Injection of horses with EIAV results in persistent infection accompanied by viremia, fever, anemia, tissue injury, and erythrocyte destruction (14, 16). The virus infects cells of the monocyte or macrophage lineage, and stress or immunosuppression may precipitate a resumption of clinical disease (13, 15). Recent studies revealed morphological and structural similarities between EIAV and HIV (23, 25).

We have recently shown that topoisomerase I (TOPO-I) activity is present in EIAV, HIV type 1 (HIV-1), and Moloney murine leukemia virus particles. In the case of EIAV, the enzyme was located in the viral cores. Furthermore, this virus-associated enzyme differs in its characteristics from the cellular TOPO-I. We have also demonstrated that the EIAV p11 nucleocapsid protein and the cellular TOPO-I share similar epitopes (21). We have found that virus-associated TOPO-I activity is inhibited by camptothecine (CPT), a known specific inhibitor of eukaryotic TOPO-I (1, 12, 24). CPT is a natural product derived from the Asian tree, *Camptotheca acuminata* (26), which has strong antitumor activity against a wide range of experimental tumors (4, 26) and human colon cancer (5). In its cytotoxic doses, CPT inhibits RNA and DNA synthesis and causes rapid and reversible fragmentation of DNA in mammalian cells (11, 24, 26). It has been shown that DNA TOPO-I is the main, if not the exclusive, target of CPT (1). In addition, we have demonstrated that noncytotoxic doses of CPT inhibit HIV-1 replication in acute infection of H9 cells (20). In this work, we extended our studies and demonstrated an inhibition of EIAV replication in chronically infected CF2Th cells (designated CF2Th/EIAV cells) by noncytotoxic doses of CPT.

* Corresponding author.
† Present address: Department of Microbiology and Immunology, Faculty of Health Sciences, Ben Gurion University of the Negev, Beer Sheva, Israel.

MATERIALS AND METHODS

Compounds. CPT and amsacrine (m-AMSA) were obtained from the Division of Cancer Treatment, Drug Synthesis and Chemistry Branch, National Cancer Institute. Stock solutions of the drugs, 10 mM in dimethyl sulfoxide, were stored in aliquots at −20°C.

Cells. CF2Th (CRL-6574) and CF2Th/EIAV cell lines were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum.

CPT treatment of CF2Th/EIAV cells. CF2Th/EIAV cells (10^6 per flask) were treated with different doses of CPT for 1 h at 37°C, washed, and resuspended in fresh medium. Reverse transcriptase (RT) activity in the culture medium was measured 48 h (or as otherwise indicated) posttreatment as described previously (6). The cells were harvested for further analysis.

Long-term CPT treatment of CF2Th/EIAV cells. CF2Th/EIAV cells (10^6 per dish) were treated with 0.02 μM CPT. Fresh medium containing CPT was added every 4 days, and cells were diluted to 10^6 cells per plate. Cell viability was determined by the trypan blue exclusion assay, and cell growth was determined by cell counting. RT activity was measured in EIAV particles isolated from the culture medium 14, 28, 42, and 52 days after the first CPT treatment.

IF assay. The effect of CPT on the EIAV p26 protein and its pr55^{gag} precursor was determined by an indirect immunofluorescence (IF) assay. CF2Th/EIAV cells (10^5 per flask) were treated with various concentrations of CPT for 24 h. CF2Th cells treated with the same CPT doses served as a control. After the incubation periods, cells were fixed with methanol-acetone (1:1), acetone, or formaldehyde for 10 to 15 min, washed with phosphate-buffered saline (pH 7.3) (PBS), reacted with EIAV p26 antiserum or with rabbit

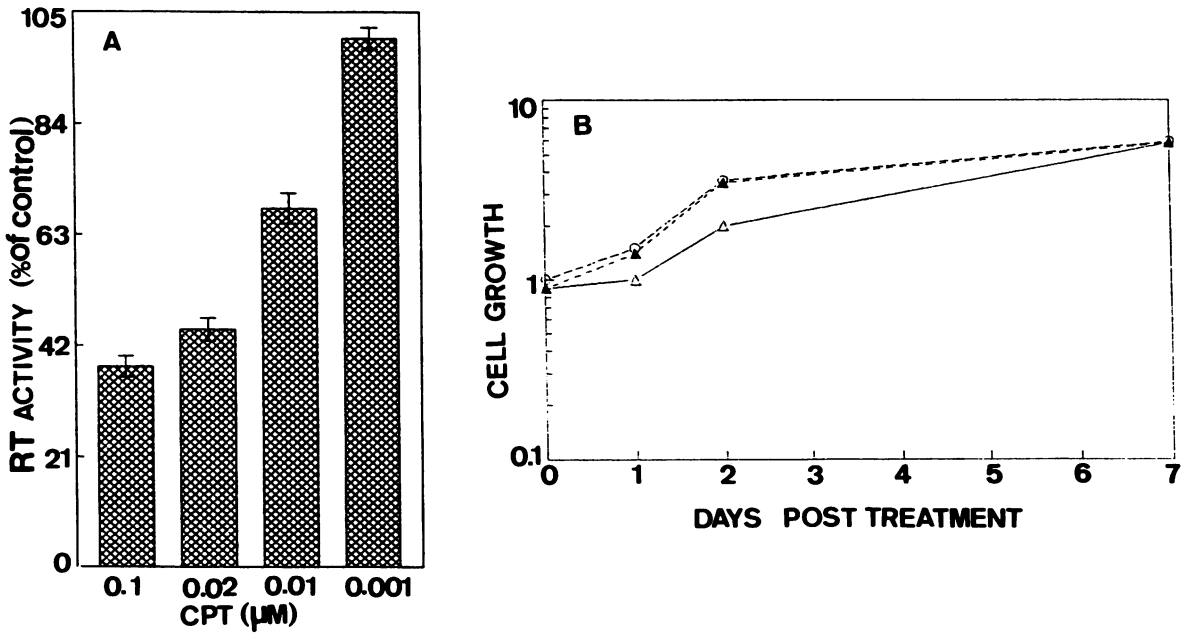

FIG. 1. Effect of 1-h CPT treatment on EIAV replication in CF2Th/EIAV chronically infected cells. Different CPT doses were added to 10^6 cells per flask for 1 h at 37°C. Cells were washed and resuspended in fresh medium. RT activity (A) was measured 48 h posttreatment, and cell growth (B) was determined 1, 2, and 7 days posttreatment as described in Materials and Methods. RT activity detected in the different CPT treatments is expressed as percentage of the RT activity observed in the untreated cells. △, 0.02 μM CPT; ▲, 0.01 μM CPT; ○, control.

normal serum for 30 min at 37°C, washed with PBS, and reacted with fluorescein-conjugated goat anti rabbit immunoglobulin G. The stained slides were washed in PBS, mounted with 90% glycerol, and examined with a fluorescent microscope. The percentage of inhibition of viral antigen expression was determined by the ratio of IF-positive cells in drug-treated cultures to IF-positive cells in infected control cultures.

RIPA. CF2Th/EIAV cells were or were not exposed to 0.02 μM CPT and radioactively labelled for 16 h at 37°C with ^{35}S-methionine (500 μCi per flask) (Amersham). Labelled cells were washed twice in serum-free Dulbecco modified Eagle medium. Sample cultures were taken for further analysis at the end of the labelling period, and the others were chased by addition of fresh medium for 48 and 72 h at 37°C (chase experiments). In addition, the culture medium from each time point was collected for virus purification. In order to precipitate viral proteins from the cells, 300 μl of radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-Cl, pH 7.5, 0.5 M NaCl, 0.5% Nonidet P-40, 0.25% Na deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) was added to the labelled cells and cells were disrupted at 4°C by repeated aspiration through a 22-gauge needle. The supernatant was clarified by centrifugation for 1 h at 4°C (100,000 × *g*, 50 Ti rotor). The supernatant (10^7 cpm) was treated overnight at 4°C with EIAV p26 antiserum (9) (1:100 dilution) or with rabbit normal serum and then incubated for 2 h at 4°C with protein A-Sepharose (0.1 g/ml of RIPA buffer). The immunocomplexes were precipitated, washed five times with RIPA buffer, and analyzed on a 10% polyacrylamide gel. EIAV was purified from the culture medium by centrifugation through a Ficoll gradient (22), and the pellet containing the virus was analyzed by immunoprecipitation with EIAV p26 antiserum as described above.

RESULTS

To investigate the effect of CPT on EIAV replication, it was first necessary to determine the appropriate dose of this drug that would inhibit replication without affecting the viability and growth rate of the host cell. For this purpose, 10^6 CF2Th/EIAV cells were treated with different CPT doses for 1 h at 37°C and washed and fresh medium was added. RT activity in the culture medium was measured 48 h after treatment, and cell viability and growth were determined 24 h, 48 h, and 7 days posttreatment. As shown in Fig. 1A, CPT at 0.01 to 0.1 μM inhibited virus replication by 32 to 52%. No effect on either cell viability (as determined by trypan blue dye exclusion) or cell growth was detected (Fig. 1B). CPT is an anticancer drug and the only known TOPO-I inhibitor, so it was interesting to compare its effect with that of another anticancer drug, the TOPO-II inhibitor amsacrine. No inhibitory effect on EIAV replication was observed with either cytotoxic or noncytotoxic doses of this drug (Table 1).

Since 1-h treatment with CPT reduced EIAV production 32 to 52%, we examined the effect of a long-term CPT treatment. For this purpose, 10^6 CF2Th/EIAV cells were

TABLE 1. Effect of amsacrine on EIAV replication in CF2Th/EIAV cells

Amsacrine (μm)	Cell growth (10^5 cells/ml)	RT activity (cpm/10^5 cells)
0	3.3	53,825
0.4	1.8	63,280
0.8	1.4	67,612
1.2	0.5	107,082
1.6	0.5	92,352
2	0.3	150,650

TABLE 2. Effect of continuous CPT treatment on EIAV replication in CF2Th/EIAV cells

Days of continuous CPT treatment	CPT	RT activity (cpm/10^6 cells) 48 h posttreatment[a]	% Inhibition[a]
14	−	151,602 ± 2,000	91 ± 2
	+	14,132 ± 500	
28	−	156,300 ± 3,500	80 ± 6
	+	33,619 ± 620	
42	−	103,223 ± 1,600	85 ± 3
	+	15,873 ± 270	
52	−	115,706 ± 1,700	90 ± 1
	+	12,639 ± 1,400	

[a] The data are means from four different experiments ± standard deviations.

treated with 0.02 μM CPT and fresh medium containing CPT was added every 4 days (as described in Materials and Methods). RT activity in the culture medium was measured 48 h after each CPT treatment. As can be seen in Table 2, 90% inhibition of EIAV replication was observed after 14 days of continuous CPT treatment, and this level of inhibition remained constant throughout the entire 52 days of subsequent CPT treatment in this experiment. No effect on cell viability or cell growth was observed during this period of CPT treatments.

Inhibition of EIAV antigen expression in CF2Th/EIAV cells by CPT. In order to determine the effect of CPT on EIAV antigen expression, we measured antigen production in treated cells by IF assays and RIPA. Control CF2Th/EIAV cells were all positive for p26 antigen in IF analysis, while cells treated with various concentrations of CPT for 24 h showed reduced levels of immunofluorescent staining; the percentage of antigen-positive cells decreased when the CPT doses were increased (Fig. 2). The reduction in IF-positive cells was 45% at 0.01 μM CPT, 60% at 0.02 μM CPT, and 85% at 0.1 μM CPT. To substantiate the IF results, we also measured the effect of CPT on viral protein expression by the immunoprecipitation assay. As shown in Fig. 3A, lane 2, CPT specifically inhibits the expression of the p26 precursor (i.e., $pr55^{gag}$), while no effect on the total protein synthesis in the cells was detected as assessed by the degree of incorporation of ^{35}S-methionine in trichloroacetic acid precipitates obtained from CPT-treated versus untreated CF2Th/EIAV cells at different days posttreatment (i.e., day 1, 318,583 versus 326,699 cpm; day 2, 208,786 versus 298,800 cpm; day 3, 360,170 versus 293,964 cpm).

Moreover, the chase experiments indicated a decrease in the labelled precursor in the untreated cells at 48 h postlabelling (Fig. 3A, lane 3), whereas no such decrease was detected during this time in the CPT-treated cells (Fig. 3A, lane 4). At 72 h posttreatment, the labelled pr55 level decreased to a very low level in both CPT-treated and untreated cells; thus, we could not detect differences between the treated and untreated cells. In parallel, we also found labelled p26 capsid protein released into the culture medium of the untreated cells but not the culture medium of the CPT-treated cells at 24, 48, and 72 h postlabelling (Fig. 3B). The results are compatible with our previous RT data, further substantiating the antiviral inhibitory effect of CPT.

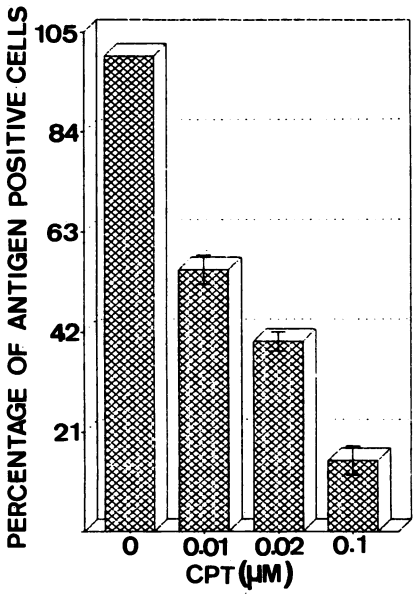

FIG. 2. Effect of CPT on p26 expression in CPT-treated and untreated cells detected by the IF assay. CF2Th/EIAV cells were or were not treated with various CPT doses for 24 h at 37°C and fixed with methanol-acetone (1:1) as described in Materials and Methods. The fixed cells were reacted with EIAV p26 antiserum or rabbit normal antiserum washed with PBS and reacted with fluorescein-conjugated goat anti-rabbit immunoglobulin G. The stained slides were examined under a fluorescent microscope. The percentage of viral antigen expression was determined by the ratio of IF-positive cells in drug-treated cultures to IF-positive cells in infected control cultures.

DISCUSSION

The *Lentivirinae* group includes causative agents of severe diseases in humans and animals, such as HIV, EIAV, and simian immunodeficiency virus. A wide variety of antiviral drugs such as azidothymidine (18) and dideoxycytidine (17) are useful as antiviral drugs because of a more efficient inhibition of viral functions than cellular ones. The presence of TOPO-I activity in retroviral particles suggests a new target for developing potential antiviral drugs. In this paper, we show that treatment with the only known TOPO-I-specific inhibitor, CPT, inhibits the replication of EIAV in chronically infected CF2Th/EIAV cells (32 to 52%) at doses which did not have any effect on cell viability. In contrast, amsacrine, which is a TOPO-II-specific inhibitor, did not have any effect on EIAV replication at noncytotoxic or cytotoxic doses. The inhibition observed with noncytotoxic doses of CPT is consistent with a specific antiviral effect rather than a result of cell toxicity, since we have shown that continuous treatment with CPT results in a 92% reduction in EIAV production in the absence of effects on cell viability. This inhibitory effect is most likely the result of reduced RNA expression, since we observed significant reductions in the levels of EIAV p26 and its precursor $pr55^{gag}$ proteins. The number of p26-positive cells, as measured by IF, was reduced 85% following CPT treatment, and a significant reduction in the intracellular level of $pr55^{gag}$ precursor was detected by the RIPA. Moreover, we could not detect the release of EIAV p26 protein in the culture media from CPT-treated CF2Th/EIAV cells. These data are compatible

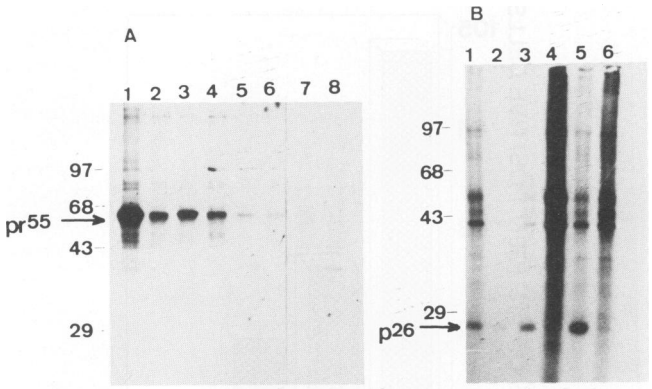

FIG. 3. Effect of CPT on EIAV p26 expression detected by RIPA. (A) CF2Th/EIAV cells were exposed to 0.02 μM CPT (lanes 2, 4, 6, and 8) or left untreated (lanes 1, 3, 5, and 7), labelled for 16 h at 37°C with ^{35}S-methionine, washed, and either analyzed immediately (lanes 1, 2, 7, and 8) or chased by addition of fresh medium for 48 h (lanes 3 and 4) or 72 h (lanes 5 and 6). Immunoprecipitations with EIAV p26 antiserum (lanes 1 to 6) or normal rabbit antiserum (lanes 7 and 8) were performed. (B) The virus was isolated from the culture medium of CPT-treated (lanes 2, 4, and 6) and untreated (lanes 1, 3, and 5) cells by using a Ficoll gradient as described in Materials and Methods. Immediately after the 16-h labelling period (lanes 1 and 2) or after chasing for 48 h (lanes 3 and 4) and 72 h (lanes 5 and 6), the viral pellet was immunoprecipitated with EIAV p26 antiserum. All of the samples in panels A and B were analyzed by electrophoresis on an SDS–10% polyacrylamide gel. Size markers are indicated in kilodaltons on the left.

with our RT results and indicate that the production of EIAV particles by chronically infected cells was inhibited by CPT.

The CPT inhibitory effect on EIAV replication is not exclusive for EIAV, since we have shown that this drug inhibits HIV replication in HIV acutely infected cells (20), as well as Moloney murine leukemia virus replication and integration (19). Thus, CPT in noncytotoxic doses may represent a new class of a general antiretroviral agent.

Agents such as RT inhibitors (7, 18), which block retroviral replication, act at early stages of the viral infection process, while other agents, such as alpha interferon, have been reported to suppress HIV expression in chronically infected cells (10). CPT appears to act as an antiviral drug at both levels and is capable under certain conditions of both blocking the initial stage of retroviral infection and reducing viral production in chronically infected cells. It thus offers potential advantages over drugs that function only on one level.

TOPO-I is the main, if not the exclusive, target of CPT (1); thus, our results suggest that the inhibitory effect of CPT on EIAV replication is likely to be due to TOPO-I inhibition. Moreover, since low doses of CPT could inhibit the expression of EIAV p26 without affecting the total protein synthesis, viral RNA synthesis may be more highly sensitive to TOPO-I inhibition than most cell genes or TOPO-I may play a more direct role in the viral protein expression. We have shown a CPT-sensitive TOPO-I activity in EIAV cores (21), and experiments are in progress in order to determine the role of TOPO-I in the EIAV life cycle.

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